Pre- and post-copulatory sexual selection in the least killifish, *Heterandria formosa*

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Academic dissertation

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Typically, the scientific paper or monograph presents an immaculate appearance, which reproduces little or nothing of the intuitive leaps, false starts, mistakes, loose ends, and happy accidents that actually cluttered up the inquiry.

(Merton, 1968)

Contents

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. Ala-Honkola, O., Friman, E. & Lindström, K. Could parent-offspring conflict explain the cost of polyandry in a poeciliid fish *Heterandria formosa*? – Manuscript.

II. Ala-Honkola, O., Uddström, A., Diaz Pauli B. & Lindström, K. 2009. Strong inbreeding depression in male mating behaviour in a poeciliid fish. – *Journal of Evolutionary Biology* 22:1396-1406.

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IV. Ala-Honkola, O., Säilä, L. & Lindström, K. Males do not prefer large females in a poeciliid fish *Heterandria formosa*. – Submitted manuscript.

Contributions

	I	II	III	IV
Original idea	OA-H, KL	OA-H	OA-H	OA-H
Study design	OA-H, KL	OA-H, KL	OA-H, KL	OA-H, KL
Data collection	OA-H, EF	AU, BDP	OA-H, LT	LS
Data analysis	OA-H	OA-H	OA-H	OA-H
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Abstract

It has been only recently realized that sexual selection does not end at copulation but that post-copulatory processes are often important in determining the fitness of individuals. In this thesis, I experimentally studied both pre- and post-copulatory sexual selection in the least killifish, *Heterandria formosa*. I found that this species suffers from severe inbreeding depression in male reproductive behaviour, offspring viability and offspring maturation times. Neither sex showed pre-copulatory inbreeding avoidance but when females mated with their brothers, less sperm were retrieved from their reproductive system compared to the situation when females mated with unrelated males. Whether the difference in sperm numbers is due to female or male effect could not be resolved. Based on theory, females should be more eager to avoid inbreeding than males in this species, because females invest more in their offspring than males do. Inbreeding seems to be an important part of this species biology and the severe inbreeding depression has most likely selected for the evolution of the post-copulatory inbreeding avoidance mechanism that I found.

In addition, I studied the effects of polyandry on female reproductive success. When females mated with more than one male, they were more likely to get pregnant. However, I also found a cost of polyandry. The offspring of females mated to four males took longer to reach sexual maturity compared to the offspring of monandrous females. This cost may be explained by parent-offspring conflict over maternal resource allocation. In another experiment, in which within-brood relatedness was manipulated, offspring sizes decreased over time when within-brood relatedness was low. This result is partly in accordance with the kinship theory of genomic imprinting. When relatedness decreases, offspring are expected to be less co-operative and demand fewer resources from their mother, which leads to impaired development.

In the last chapter of my thesis, I show that *H. formosa* males do not prefer large females as in other Poeciliidae species. I suggest that males view smaller females as more profitable mates because those are more likely virgin. In conclusion, I found both pre- and post-copulatory sexual selection to be important factors in determining reproductive success in *H. formosa*.

Summary

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1. Introduction

In his classic study, Bateman (1948) argued that the reproductive success of a male depends on the number of females he mates with, but that females should not actively seek extra copulations after a successful one, because they get enough sperm to fertilise all their eggs in one copulation. However, molecular markers have revealed that in almost all species studied in this respect, females mate with many males during one reproductive season (Birkhead & Møller 1998). But why do females mate with more than one male? This question has puzzled biologists, because mating is costly, and it has been under very intense study during the last ten years. In September 2009, the review article by Jennions and Petrie (2000) on genetic benefits of polyandry (female multiple mating) had been cited over 500 times.

The possible benefits of polyandry can be divided into direct (material) and indirect (genetic) benefits (Table 1). If females gain direct benefits, such as nuptial food gifts that males of some insect species provide, the evolution of polyandry is easy to understand. Females that mate with many males get more food and produce more offspring (Arnqvist & Nilsson 2000). However, Jennions and Petrie (2000) argue that multiple mating solely for direct benefits is unlikely, because it also leads to the possibility of genetic benefits. Even so, it has proven to be difficult to show that females gain genetic benefits from polyandry (Simmons 2005). There is evidence that polyandry increases egg hatching success in insects (meta-analysis by Simmons 2005) and reduces early reproductive failure in mammals (comparative study of polytocous mammal species by Stockley 2003). Effects of polyandry on offspring reproductive success have been studied in five experiments. Daughters of polyandrous females produced more offspring in the bulb mite (*Rhizoglyphus robini*) (Konior et al. 2001), but in the red flour beetle (*Tribolium castaneum*) daughters of polyandrous females had reduced fitness in conditions where intraspecific competition was high (Pai & Yan 2002). In contrast, sons of polyandrous females in *T. castaneum* had enhanced fitness in two studies (Bernasconi & Keller 2001; Pai & Yan 2002). Klemme et al. (2008) showed that sons of polyandrous bank vole females produced more offspring than sons of monandrous females in a semi-natural setting, but in the black field cricket (*Teleogryllus commodus*),

sons of polyandrous females had reduced reproductive success (Jennions et al. 2007). Thus the fitness effects of polyandry are very variable.

Table 1. Direct (material) and indirect (genetic) benefits that females may gain from multiple mating (Keller & Reeve 1995; reviewed by Reynolds 1996; Møller 1998; Jennions & Petrie 2000). The numbering after the indirect benefits refers to the order in which the specific benefits are discussed in section 1.1.

Direct benefits	Indirect benefits
Higher probability of fertilisation	Genetically more resistant offspring (1)
Supply of functional sperm	Genetically more diverse offspring (1)
Nuptial food gifts	Ensuring genetic compatibility (2)
Lower probability of infanticide	Sons that are good sperm competitors (3)
Parental care from additional males	Genetically more viable offspring (3)
Prospecting for future males	Compensation for a mate of low quality
Ejaculate nutrients	
Lower probability of sexual harassment	
Substances that promote egg maturation and oviposition	

When females mate with more than one male, an opportunity for sperm competition between the ejaculates of different males arises (Parker 1970; Parker 1998). According to sperm competition models, the increased risk of sperm competition (meaning that a higher proportion of females mates with two males) leads to an increased ejaculate expenditure per mating opportunity across species (Parker 1998). Comparative studies on for example primates (Harcourt et al. 1981), bats (Hosken 1997), butterflies (Gage 1994) and fish (Stockley et al. 1997) support this prediction: as the risk of sperm competition increased, relative testis size increased in all these groups. Hosken and Ward (2001) first showed experimentally that increased sperm competition leads to increased sperm investment (larger testis size) in the yellow dung fly (*Scathophaga stercoraria*).

Also, a number of sperm quality traits have been shown to be important in determining sperm competition success. Sperm motility is important as faster swimming sperm have been shown to be better at fertilising eggs in the domestic fowl (*Gallus gallus domesticus*) (Birkhead et al. 1999) and Atlantic salmon (*Salmo salar*) (Gage et al. 2004). In species with ameboid sperm, larger sperm have been shown to be more successful in sperm competition in the bulb-mite (*Rhizoglyphus robini*) (Radwan 1996) and the *Caenorhapditis elegans* nematode (LaMunyon & Ward 1998). Sperm competition may also select for more viable sperm, as in insects, polyandrous species had more viable sperm (measured as the

proportion of live sperm) as compared to monandrous species (Hunter & Birkhead 2002). In a recent study on 29 species of closely related cichlid fishes, Fitzpatrick et al. (2009) showed that sperm competition selects for increases in the number, size and longevity of sperm.

In addition to sperm competition, females may also affect fertilisation success of males. Eberhard (1996) defined cryptic female choice as "a female-controlled process or structure that selectively favours paternity by conspecific males with a particular trait over others that lack the trait when female has copulated with both types." The problem with this definition is that it excludes passive bias of paternity due to for example differences in the size of sperm storage sites among females (Pitnick & Brown 2000). Thus, Pitnick and Brown (2000) suggest a different definition of cryptic female choice: "non-random paternity biases resulting from female morphology, physiology or behaviour that occur after coupling."

Edvardsson and Arnqvist (2000) were the first to experimentally demonstrate cryptic female choice by manipulating the perceived male quality in red flour beetles (*Tribolium castaneum*). They showed that females bias paternity of males based on the copulatory courtship behaviour (leg rubbing) that males perform during copulation. Since then, several possible behavioural mechanisms of cryptic female choice have been identified: in *T. castaneum* female copulatory behaviour (quiescence) affects sperm transfer (Bloch Qasi 2003), in a spider (*Argiope keyserlingi*), females adjust the timing of sexual cannibalism (Elgar et al. 2000), in the domestic fowl, females eject sperm of unpreferred subdominant males more often than sperm of preferred dominant males (Pizzari & Birkhead 2000), in the guppy (*Poecilia reticulata*), the copulation duration, which is thought to be at least partly under female control, correlates with the amount of sperm retrieved from the female's gonoduct (Pilastro et al. 2007) and in the black field cricket (*Teleogryllus commodus*), females adjust the timing of spermatophore removal (Bussiere et al. 2006).

In addition to behavioural mechanisms, sperm-female interactions are a potential mechanism of cryptic female choice. Several studies have shown that male-female interactions affect paternity patterns (Lewis & Austad 1990; Wilson 1997; Clark et al. 1999, 2000; Birkhead 2004). For example, in the ascidian *Diplosoma listerianum*, the female oviduct selects which sperm reach the ovary; self-fertilisation and certain crosses are prevented by this mechanism of sperm selection (Bishop 1996; Bishop et al. 1996). In the sea urchin genus *Echinometra*, eggs are preferentially fertilised with sperm that have a similar genotype for a certain protein as the eggs (Palumbi 1999). Also the major histocompatibility complex (MHC) might be involved in non-random fertilisation in vertebrates (Wedekind et al. 1996; Olsson et al. 2004). An interaction between female sperm storage organ (seminal receptacle) size and sperm length has been shown to be

an important factor in male sperm competition success in *Drosophila melanogaster* (Miller & Pitnick 2002).

1.1 Genetic benefits of polyandry

Several genetic benefits have been proposed to explain polyandry (Table1, Jennions & Petrie 2000). Next, I will introduce in more detail those hypotheses that are the most relevant to my study.

(1) Polyandry for offspring diversity

Sherman et al. (1988) suggested that in social insects, queens are polyandrous in order to increase the genetic diversity of workers, because it increases parasite and pathogen resistance. Several studies have supported this hypothesis (for example Liersch & Schmid-Hempel (1998); Baer & Schmid-Hempel (1999); Tarpy & Seeley (2006)). Another way in which genetic diversity within broods could be beneficial, is if environmental conditions are so unpredictable that females can not predict the best genotype of a male, but mate with many males in order to increase the chance that there is a male with the best possible genotype among her partners (Yasui 1998) (i.e. bet-hedge: decrease their mean fitness but also reduce the variance of it). In unpredictable conditions, offspring diversity within a brood might increase the chance that some of them survive (Barton & Post 1986). However, the conditions that favour the evolution of polyandry for offspring diversity in unpredictable environmental conditions are very restrictive: i) genetic bet-hedging should be effective, which requires small population size (see example in Sarhan & Kokko 2007), ii) full-sibs should compete for resources more intensely than half-sibs and iii) half-sibs should interact cooperatively (Yasui 1998).

(2) Polyandry to ensure genetic compatibility

Zeh and Zeh (1996, 1997) proposed that polyandry may have evolved as a measure for females to avoid fertilising their eggs with sperm of genetically incompatible males. Incompatibility between mates may be caused by a number of factors, for example cellular endosymbionts (e.g. *Wolbachia*), transposable elements and the break down of coadapted gene complexes (Zeh & Zeh 1996; Tregenza & Wedell 2000). Stockley et al. (1993) first suggested that females are polyandrous in order to avoid inbreeding. Inbreeding can be seen as one type of genetic incompatibility if it causes inbreeding depression (Box 1) and it is also the easiest to test empirically (Tregenza & Wedell 2000). Several studies have tested the hypothesis that when females are mated to males that differ in relatedness, the less related one sires more offspring (Table 2). Some studies have supported it, whereas others have not (Table 2). Female ability to avoid inbreeding sometimes depends on the mating order (Table 2).

However, theory predicts that it may not always be adaptive to avoid inbreeding, because the negative effects of inbreeding may be overridden by benefits brought about by kin selection (Parker 1979; Kokko

& Ots 2006). Males and females may also have different thresholds to avoid inbreeding. In many species females continue to invest in their current offspring after fertilisation, while males are ready to mate again with other females. Such differences in parental investment will result in a situation where females are less tolerant to inbreeding than males (Parker 1979; Kokko & Ots 2006). In such species, according to theory, males should avoid mating with sisters if fitness loss due to inbreeding depression is more than two-thirds whereas females should avoid mating with brothers when fitness loss is more than one-third (Parker 1979; Kokko & Ots 2006). A conflict over incestuous mating arises when the magnitude of inbreeding depression is such that females should avoid mating with siblings but males should not (Parker 1979).

Table 2. Studies that have tested the hypothesis that less related males sire more offspring in sperm competition and used molecular markers for paternity analyses.

Study and species	Experiment	Relatedness of mates	Less related males sire more offspring
Olsson et al. 1996, sand lizard	No	Unknown	Yes
Stockley 1997, common shrew	No	Unknown	No, epididymal sperm count important
Kraaijeveld-Smit et al. 2002, *Antechinus agilis*	Yes	Unknown	Yes, but mating order more important
Mack et al. 2002, *Drosophila melanogaster*	Yes	Sib, half-sib, cousin/ unrelated	Yes
Garner & Schmidt 2003, alpine newt	Yes	Unknown	Yes
Bretman et al. 2004, *Gryllus bimaculatus*	Yes	Sib/non-sib	Yes, when non-sib mates first
Denk et al. 2005, mallard	Yes	Sib/non-sib	No, sperm motility important
Simmons et al. 2006, *Teleogryllus oceanicus*	Yes	Sib/non-sib	Yes, when non-sib mates first
Teng & Kang 2007, *Locusta migratoria*	Yes	Sib/non-sib	No, mating order important
Lane et al. 2007, red squirrel	No	Unknown	No
Firman & Simmons 2008, house mouse	Yes	Sib/non-sib	Yes
Sherman et al. 2008, *Litoria peronii* (a frog)	Yes	Unknown	No, more related males sire more offspring
Evans et al. 2008, guppy	Yes	Cousin/ unrelated	No

Box 1. Inbreeding depression.

Mating between close relatives often leads to a decrease in fitness known as inbreeding depression. The magnitude of inbreeding depression varies among species, populations, environments and traits (Keller & Waller 2002). It has been suggested that inbreeding depression may be more severe in stressful environments (Roff 1997), but this is not a general rule (Armbruster & Reed 2005). There are two hypotheses, which aim to explain inbreeding depression (Charlesworth & Charlesworth 1987). According to the overdominance hypothesis, inbreeding depression is due to heterozygote advantage. Inbreeding increases homozygosity and therefore decreases the frequency of heterozygotes that are assumed to be superior to homozygotes. This leads to a decline in fitness. Alternatively, the partial dominance hypothesis proposes that inbreeding depression is caused by homozygosity of deleterious recessive alleles, which decreases the fitness of inbred individuals. Current empirical evidence suggests that partial dominance is the most important mechanism of inbreeding depression (for example Roff 2002; Swindel & Bouzat 2005; Fox et al. 2008) but in some systems inbreeding depression is caused by overdominance (Kärkkäinen et al. 1999).

The magnitude of inbreeding depression is often larger in traits closely related to fitness, such as survival and fecundity, as compared to morphological traits (Falconer 1989; DeRose & Roff 1999). This is because the relative proportion of dominance variance (one component of non-additive genetic variance) is expected to be greater in such traits, as additive genetic variance has been decreased by directional selection (Mousseau & Roff 1987; Roff 1997, but see Price & Schluter 1991 and Houle 1992 for different views). Roff & Emerson (2006) present some evidence that the ratio of non-additive to additive genetic variance is higher in life-history traits compared to morphological traits. Of the non-additive genetic variance, directional dominance variance is a prerequisite for inbreeding depression to occur (Lynch & Walsh 1998).

(3) Polyandry for good genes

Sivinski (1984) was the first to suggest that polyandrous females could produce offspring of higher viability than monandrous females, if a male's sperm competitive ability was correlated with his offspring's competitive ability. Madsen et al. (1992) first found correlational evidence for this. Yasui (1997) showed theoretically in his "good sperm" model that polyandrous females could benefit through more viable offspring if males with better sperm competitive ability posses genes of higher general viability. The difference between the "good sperm" (also called "the intrinsic male quality") hypothesis and the genetic compatibility hypothesis is that in the first case, there is one male that is the best option for all females, instead of a compatible male that is different for each female type. The genetic incompatibility hypothesis requires non-additive genetic variation, whereas the intrinsic male quality hypothesis requires

additive genetic variation in fitness (Neff & Pitcher 2005).

Fisher et al. (2006) were the first to find sound empirical support for the "good sperm" hypothesis: males with more competitive ejaculates sired more viable offspring in a small marsupial (*Antechinus stuartii*). Another testable prediction of the "good sperm" model is that the trait that determines fertilisation success in males is genetically correlated with the mechanism that females use to bias paternity (Evans & Simmons 2008). Simmons and Kotiaho (2007) found support for this prediction in the dung beetle (*Onthophagus taurus*), providing the best evidence so far for the "good sperm" process. In dung beetles, the trait that determines fertilisation success in males is sperm size and the mechanism that females use to bias paternity is the size of the spermatheca (García-Gonzalez & Simmons 2007).

Simmons (2005) argues that the reason for scarce empirical evidence in support of the "good sperm" hypothesis is due to the fact that only recently suitable experiments to show it have been performed. Confounding maternal effects and the difficulty of estimating fertilisation success before embryo mortality make it difficult to distinguish between the genetic compatibility and the "good sperm" hypothesis (Simmons 2005; García-González 2008). Recently, a quantitative genetic approach has been used to show that males differ in their ability to sire offspring with high embryo viability (García-González & Simmons 2005). In fact, several studies have now shown that the "good sperm" and the compatibility hypotheses are not mutually exclusive, but that both processes can simultaneously act at fertilisation and during offspring development (Wedekind et al. 2001; Evans et al. 2007; Ivy 2007).

1.2 Non-adaptive polyandry

In addition to adaptive hypotheses of female multiple mating, Halliday and Arnold (1987) proposed a non-adaptive hypothesis. They suggested that selection on male mating tendency leads to a correlated response in females. This requires that selection on mating tendency is stronger on males than females. So far one study has supported this hypothesis: in the burying beetle (*Nicrophorus vespilloides*), mating frequency and mating speed were positively genetically correlated between males and females (House et al. 2008). However, several other studies have not supported this hypothesis (Sgro et al. 1998, *Drosophila melanogaster*; Grant et al. 2005, the stalk-eyed fly *Cyrtodiopsis dalmanni*; Harano & Miyatake 2007, the adzuki bean beetle *Callosobrochus chinensis*). A few earlier studies on the subject suffered from various limitations that may have affected interpretations of the results (Arnold & Halliday 1992; Butlin 1993).

The other non-adaptive hypothesis, the sexual conflict hypothesis, is also based on the assumption that selection on mating tendency is stronger on males than females. Such selection can favour traits that increase male mating success even if those traits

harm females (Parker 1979; Rice 1996; Chapman et al. 2003; Arnqvist & Rowe 2005). However, females may evolve counter-adaptations that reduce male-induced harm, which may lead to an antagonistic co-evolutionary arms race between sexes (Parker 1979; Holland & Rice 1998; Gavrilets et al. 2001). Empirical support for the sexual conflict hypothesis comes for example from studies that have shown that under strict monogamy, males evolve to be less harmful to females (Holland & Rice 1998; Martin & Hosken 2003).

1.3 Cost of mating

The reason why female multiple mating is so puzzling is that mating is often (if not always) costly. First of all, it takes time and energy, but it can also make females more susceptible to predators and parasite or microbial infections (Daly 1978; Rowe 1994). In the meal worm beetle (*Tenebrio molitor*), mating reduces immune function (Rolff & Siva-Jothy 2002). Some seminal fluid products that males transfer during copulation are harmful to females and decrease their life-span (Chapman et al. 1995). In addition, specific structures in male copulatory organs may injure females during copulation (Crudgington & Siva-Jothy 2000; Stutt & Siva-Jothy 2001; Blanckenhorn et al. 2002).

Polyandry itself may create costs that are related to genetic differences among the males that a female mates with (see Box 3). In the leaf-cutting ant (*Atta colombica*), queens that had stored sperm from many mating partners had a weaker immune response than queens that had stored a similar amount of sperm from fewer partners, implying a cost of storing genetically different ejaculates (Baer et al. 2006). When broods have multiple sires, the decrease in within-brood relatedness may make the offspring less cooperative than broods with only one sire (Hamilton 1964), as shown by Evans and Kelley (2008) in the shoaling behaviour of guppies. In viviparous species, a conflict of interest between maternal and paternal genomes arises during the gestation period (review in Crespi & Semeniuk 2004). If broods have multiple sires, a conflict may also arise between different paternal genomes (see Box 3).

Box 2. Parent-offspring conflict.

The optimal amount of parental investment is different for a parent and its offspring, because the offspring is more related to itself than it is to the parent and the parent is more related to itself than to its offspring (Trivers 1974). A parent is selected to leave resources for its future offspring and thus an offspring maximises its fitness at a higher parental investment than the parent maximises its own fitness. This discrepancy in optimal parental investment leads to parent-offspring conflict. Hamilton's rule predicts that offspring also disagree among themselves about the amount of parental investment they should get (Hamilton 1964). Each offspring values its own inclusive fitness higher than that of its siblings and is expected to seek more than its fair share of maternal resources (Hamilton 1964; Wells 2003). As females often mate with different males during the same reproductive season or between different seasons, also paternally and maternally inherited genes in the offspring have different optima of parental investment. Paternal alleles are selected to extract more resources from their mother than the mother is willing to give as she is selected to save resources for her future offspring (Haig & Westoby 1989; Moore & Haig 1991). The mother's future offspring are of no interest for the paternal allele if the female mates with a different male during the next reproductive event. In its extreme, this conflict between maternally and paternally inherited genes in the offspring over parental care, may lead to genomic imprinting (Haig 2000). The activity of an imprinted gene depends on its parent of origin (Haig 2000).

Traditionally, parent-offspring conflict has been modelled with begging level of chicks of birds in mind (Mock & Parker 1997). That is because begging may be the easiest to study in this context. The models of intrabrood competition predict that the loudness of begging increases as the within-brood relatedness decreases (Macnair & Parker 1979; Parker & Macnair 1979), because offspring selfishness increases when relatedness decreases (Hamilton 1964). This result has been confirmed in an experimental study on barn swallows (Boncoraglio & Saino 2008). Haig (1996) modelled the production of a hypothetical placental hormone that increases the nutrient content of maternal blood and predicted how much hormone maternal and foetal genes are selected to produce given variation in mating system, litter-size and parental origin of the foetal genes. The difference between begging calls and placental hormone production is that in the latter case, the mother is not likely to know who is begging. Placental hormones circulate in maternal blood streams and "cannot convey specific information about individual embryos, but only aggregate information about the litter as a whole" (Haig 1996). Thus parent-offspring conflict decreases offspring growth in multiply sired litters when begging means an altruistic production of hormones that increase nutrient supply from the mother.

1.4 Male choice

Male choice is much less studied than female choice. However, males are expected to exhibit choice over mating partners when females differ in fecundity, the operational sex ratio is female biased or the risk of sperm competition differs between females (Parker 1983; Anderson 1994). The most commonly observed male mating preferences are those that maximize a male's expected fertilisation success in each mating (Bonduriansky 2001). Such preferences tend to favour female phenotypes associated with high fecundity or reduced sperm competition intensity (Bonduriansky 2001). Male choosiness is expected to increase when male parental investment increases as shown by Simmons (1992) and Gwynne (1993). However, if the variance in female quality is large, males can be choosy even if their investment in each mating is insignificant compared to females' investment (Parker 1983).

The family Poeciliidae is an example of fishes in which males have a low parental investment (males only provide sperm) (Farr 1989). Despite the low parental investment, male preference for large females has been found in all species studied in this respect. The species in question are the eastern mosquito fish (*Gambusia holbrooki*) (Bisazza et al. 1989; Hoysak & Godin 2007; but see McPeek 1992), the sailfin molly (*Poecilia latipinna*) (Ptacek & Travis 1997; Gabor 1999), the guppy (*Poecilia reticulata*) (Dosen & Montgomerie 2004a; Herdman et al. 2004), the Atlantic molly (*Poecilia mexicana*) (Plath et al. 2006) and the western mosquito fish (*Gambusia affinis*) (Deaton 2008). In *Brachyrhapsis rhabdophora* only large males prefer large females (Basolo 2004). In poeciliid fishes, as in fish in general, fecundity increases with size (Bagenal & Braum 1978; Travis et al. 1990; Herdman et al. 2004), and thus males mating with large females gain benefits in terms of offspring number or quality.

In addition to fecundity, males may also differentiate females based on their expected success in sperm competition as demonstrated by Schwagmeyer and Parker (1990) in the thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). Dosen and Montgomerie (2004b) showed that male guppies are sensitive to the risk of sperm competition and prefer to associate with females that have not been recently inseminated. Also eastern mosquito fish males react to the perceived risk of sperm competition (Wong & McCarthy 2009). In insects, males have been shown to prefer virgin (Lewis & Iannini 1995: *Tribolium castaneum*; Carazo et al. 2004: *Tenebrio molitor*) or young (Simmons et al. 1994: *Requena verticalis*) females to maximise their share of paternity.

2. Aims of the thesis

This thesis has the following aims:

1) To test the effect of polyandry on female fitness by manipulating i) the number of mates a female mates with and ii) the genetic constitution of the brood a female carries when the

number of mates is kept constant but the relatedness of males differs (**I**). So far experiments examining the effects of polyandry on female fitness have been mainly performed on insects. In viviparous animals, the parent-offspring conflict is likely to arise and may affect offspring provisioning in multiply sired broods. However, this aspect has not been previously studied.

2) To measure the severity of inbreeding depression in male reproductive behaviour and several life-history traits (**II**), because not much is known about the effects of inbreeding on behaviour. Reproductive behaviour is likely to be closely related to fitness and is therefore expected to show inbreeding depression.

3) To test if females are polyandrous in order to avoid inbreeding (**III**). Previously most studies have used only offspring paternity at birth as a measure of inbreeding avoidance. I want to include a measure for inbreeding avoidance after copulation but before fertilisation (stored sperm). Based on the results from study **II**, I will see how the severity of inbreeding depression relates to the evolution of inbreeding avoidance mechanisms. The severity of inbreeding depression is known for very few of those species in which inbreeding avoidance has been assessed.

4) To study male mate choice in relation to female size, female mating status and the paternity pattern of females (i.e. do females predominantly produce offspring of the first or the second male to mate) (**IV**).

3. Study species

Heterandria formosa, the least killifish, is a small (1.3 to 4 cm) live-bearing poeciliid fish found in a variety of habitats in the coastal plain of the south-eastern USA (Martin 1980). Fertilisation is internal and the ovary of the least killifish is a single sac-like oval structure dorsally suspended in the body cavity, as in other poeciliid fishes (Fraser & Renton 1940). The trait that makes these fish different from many other related species is matrotrophy, which means that females provide embryos with resources for development through a placenta-like structure (Fraser & Renton 1940; Scrimshaw 1944), resembling the mammalian offspring provisioning. The maternal-foetal interface consists of the maternal ovarian follicle and the pericardial sac of the offspring (Grove & Wourms 1991; 1994). The offspring of one female can be seen to form one large continuous brood, as females simultaneously carry up to six broods of different developmental stages (Travis et al. 1987) (called superfetation) and thus a large number of offspring simultaneously share the maternal environment. Because of superfetation, females give birth to several young every few days and they gestate their whole reproductive life. This is another trait that makes the least killifish different from most poeciliid fishes.

The least killifish has a resource free mating system (males provide sperm only) and the reproductive behaviour of males consists mainly of forced copulations, so called gonopodial thrusts (Farr 1989; Bisazza & Pilastro 1997). During a copulation attempt, the male swings his gonopodium (intromittent organ) forward, approaches the female from behind and tries to reach the female genital opening (gonopore) with the tip of the gonopodium. Females rarely cooperate with males at mating (personal observation). Even though courtship behaviour has not been described in this species (Farr 1989), Aspbury and Basolo (2002) report "courtship-like" behaviour in their study on female choice in the least killifish. I also commonly observed behaviour that looks like courtship, and therefore I included measures of "courtship-like" behaviour in my study. Females of this species have been shown to prefer large males when given a choice between a small and a large male (Aspbury & Basolo 2002), but large males had a better mating success (measured as the number of successful copulations divided by the total number of copulation attempts) only when they mated first. Thus female preference for large males did not lead to a higher mating success of those males (Aspbury & Basolo 2002). An unanswered question is whether preferred males have a higher paternity success than unpreferred males.

In nature, least killifish populations often have high densities (Leips & Travis 1999). However, population sizes have been shown to collapse during droughts and recover slowly (Ruetz et al. 2005). As re-colonisation of sites after a drought is primarily from reproduction by surviving individuals and not by migrants (Ruetz et al. 2005), mating between relatives is quite likely to occur. Hence, inbreeding and possibly inbreeding depression may be an important part of this species' biology. In the least killifish, females should be the choosier sex in terms of inbreeding avoidance, because females make a substantial investment in the developing embryos whereas males provide sperm only. Sperm choice may be the most appropriate form of female choice in this species, because females are often unable to perform pre-copulatory choice but are most of the time forcibly inseminated (Farr 1989; Bisazza & Pilastro 1997; Birkhead 1998).

4. Material and methods

All studies in this thesis were experimental. I performed them in the aquarium facilities of the Department of Biological and Environmental Sciences, University of Helsinki. The experimental fish were aquarium born offspring of fish collected from two field locations, the Saint Johns River system and the Otter Creek river in Florida, US. The original sizes of the laboratory populations were about six hundred fish for the Saint Johns population and eight hundred fish for the Otter Creek population. The fish were maintained in several mixed-sex stock tanks (40 l) at a 14:10 light:dark photoperiod at 28 ^{o}C and fed *ad lib* twice a day with frozen *Artemia*

in the morning and frozen mosquito larvae in the afternoon. Newborn offspring were collected from stock tanks in order to raise virgin females. When males started to develop the gonopodium, they were moved to separate tanks. Similarly, females were moved to separate "virgin female" tanks when they developed black spots that indicate sexual maturity (Fraser & Renton 1940) on their anal fin and around their gonopore.

For the studies **II** and **III**, I needed to have sibling fish. These were created by allowing randomly selected pairs of virgin females and stock tank males to mate. Their offspring were raised as family groups. When the offspring started to mature, they were separated into different aquaria according to sex. Virgin females of a family were kept together in one aquarium. All the males of a family were housed together with two unrelated females in one aquarium. This ensured that the males were sexually active and producing sperm when used in the experiments. In the related guppy, the presence of females increases sperm production of males (Bozynski & Liley 2003).

Mate choice tests

I performed dichotomous mate choice test to assess possible pre-copulatory mate preferences of males and females. In study **III** I used this method to examine whether males or females avoid their siblings as mates pre *copula*, and in study **IV** to test whether males prefer females of certain size or mating status. This method is commonly used in mate choice studies in Poeciliidae (Houde 1997).

In short, in the dichotomous mate choice test the focal fish was put in the centre section of an aquarium, which was divided into three compartments using opaque dividers. The two stimulus fish were enclosed in small transparent containers and placed in each end compartment. To allow possible olfactory cues, the lids of the containers were patterned with small slits (width 1 mm) covering about 40 % of the lid area (**III** and **IV**). In study **III**, small holes were drilled (diameter 2 mm) into the walls of the containers (6 per side) to increase the water flow between the container and the aquarium. All fish were left to acclimatize for 5 minutes. After this, the dividers were removed and the focal fish was free to swim anywhere in the aquarium, but not inside the containers housing the stimulus fish. The behaviour of the focal fish was observed for 2 x 15 minutes. After the first observation period, the focal fish was again enclosed in the middle section by replacing the dividers, and the positions of the containers holding stimulus fish were switched. This was done in order to control for possible side preferences of focal fish. After this the dividers were again removed and the behaviour was observed for another 15 minutes. Mate preference was defined as the time the focal fish was interacting with the stimulus fish.

Paternity analysis with DNA microsatellite markers

In studies **I**, **II** and **III**, I performed paternity tests with DNA microsatellite markers. I used four loci developed for related species that had been tested and optimized for the least killifish (see Soucy & Travis 2003; Nakamura 2001).

5. Main results and discussion

5.1 Costs and benefits of polyandry in the least killifish

I found both benefits and costs of polyandry in the least killifish (**I**). Females mated to multiple males were more likely to become pregnant (Table 3). In the 'single-male' treatment a remarkably large proportion of females remained non-fertile. However, offspring of females mated to four males took longer to reach sexual maturity than offspring of females mated to one male, implying a cost of polyandry.

Table 3. The proportion of females producing offspring in the multiple mating experiment.

Treatment	One male	Two males	Four males
Proportion of females producing offspring	7/19 (37 %)	12/17 (71 %)	16/18 (89 %)

The aim of the second experiment of this study was to manipulate within-brood relatedness to uncover possible parent-offspring conflict over offspring provisioning. All females were mated to three males, but the males differed in relatedness. In the 'relatedness' treatment, the males were full-siblings (but unrelated to the female) and in the 'diversity' treatment, the three males were unrelated to each other and to the female.

In this 'diverse *versus* related broods' experiment, I found that females mated to three unrelated males had a shorter life span than females mated to three siblings. However, this effect was only seen at such high age that females do not normally reach in nature. The other aspect, in which these treatments differed, was how offspring size at birth changed over time. In the relatedness treatment offspring size at birth was independent of birth order, whereas in the diversity treatment later-born offspring were smaller. This finding is partly in accordance with Haig's (1996) model of placental hormone production. In that model, all litter members share the benefits of the hormone production, but the costs of the production are borne by individual offspring. Litters that produce more hormone raise fitter offspring but when the relatedness within litters decreases, the cooperation between offspring decreases as the number of potential freeloaders increases. Thus decreased relatedness within litters leads to decreased hormone production and decreased size of newborn offspring. The problem with this interpretation of my result is that there is initially no difference in offspring sizes

between treatments, but only an interaction effect.

An alternative explanation to the decrease in offspring size over time is that females invest more in early offspring when offspring are genetically diverse. Because investment is costly, fewer resources are left to be invested in the later-born offspring. In addition, this costly investment may also lead to earlier death of those females.

Earlier studies on the least killifish have found evidence for both offspring control of maternal provisioning (Schraeder & Travis 2008) and maternal control of provisioning (Leips et al. 2009). When Schraeder and Travis (2008) crossed fish from a highly polyandrous population and a nearly monandrous population, they found that offspring of males from the more polyandrous population extracted more resources from their mother than offspring of males from the nearly monandrous population, in which the parent-offspring conflict is expected to be smaller. On the other hand, Leips et al. (2009) found that at high population density, females produced larger offspring than at low density, presumably because it is better to have fewer large offspring when intra-specific competition is high, implying an adaptive maternal response on offspring size.

However, the 'higher investment in diverse broods' interpretation does not explain why females mated to four males produced offspring with longer maturation time in the multiple mating experiment. This finding could be explained by the lower demand of maternal resources by the genetically unrelated litter members, as predicted by Haig (1996). It is possible that offspring in the four-male treatment were less developed than offspring in the single-male and two-male treatments, even if there was no difference in offspring size at birth.

5.2 Inbreeding depression and inbreeding avoidance in the least killifish

Inbreeding depression

I found strong inbreeding depression in male reproductive behaviour (**II**). Inbred males performed less copulation attempts than outbred males. The standardized coefficient of inbreeding depression (Lande & Schemske 1985) for this behaviour was $\delta = 0.38$. This trait is closely related to fitness, as the number of copulation attempts correlated with paternity success. Only few other studies have looked at the effects of inbreeding on male reproductive behaviour. In *Drosophila montana*, inbreeding decreases courtship song frequency (Aspi 2000); in song sparrows (*Melospiza melodia*), it decreases courtship song repertoire (Reid et al. 2005); and in guppies (*Poecilia reticulata*), it decreases courtship behaviour (van Oosterhout et al. 2003; Mariette et al. 2006). Thus our result is in line with the earlier studies suggesting that reproductive behaviour is susceptible to inbreeding depression.

I also found that two important life history traits showed inbreeding depression. Inbred females gave birth to a lower proportion of live offspring ($\delta = 0.06$) and inbred

offspring took longer to mature (δ = 0.19 for males and δ = 0.14 for females). Increases in age at maturity and in juvenile mortality are predicted to have large impacts on fitness (Stearns 1992).

The level of inbreeding depression that I found might be strong enough to select for the evolution of inbreeding avoidance mechanisms. Theoretically, if fitness loss due to inbreeding depression exceeds δ = 1/3, females should prefer unrelated males over brothers as their mates (Parker 1979, Kokko & Ots 2006). On the other hand, males should avoid mating with sisters only if the fitness loss due to inbreeding depression is more than 2/3 (Parker 1979, Kokko & Ots 2006). Inbreeding depression in male gonopodial activity (δ = 0.38) exceeds the threshold value 1/3, at which females should avoid their brothers as mates, and inbreeding depression in male and female maturation times is also high (δ = 0.19 and 0.14). However, from these values it is difficult to estimate what the actual fitness loss of a female mating with a brother is. In any case, it certainly comes close to the threshold where she should avoid mating with her brother. A female mating with a brother would most likely produce male offspring with low reproductive success for at least three reasons. Firstly, inbred males would have a lower probability to reach maturity because of their longer development time compared to outbred males (this also applies to female offspring). Secondly, their lower mating activity would most likely lead to poor paternity success. Thirdly, their smaller size as compared to outbred males (**II**) may make them less desirable mating partners as females of the least killifish have been shown to prefer larger males (Aspbury & Basolo 2002).

Inbreeding avoidance

Based on the severity of inbreeding depression in the least killifish, it seems that inbreeding avoidance mechanisms might have been favoured over the course of this species' evolution. I measured inbreeding avoidance at three stages of reproduction: i) before copulation, ii) after copulation but before fertilisation and iii) at birth. I found that neither males nor females discriminated against their siblings in a dichotomous pre-copulatory mate choice test. An interesting finding was that when females had mated with their brothers, less sperm could be flushed from their reproductive system than when they had mated with a non-sibling. This suggests that an inbreeding avoidance mechanism has evolved in this species. However, when I compared the paternity success of brothers and unrelated males, I found no difference in the proportion of offspring sired.

Why did I not find a difference in the paternity success of brothers and unrelated males even though there was a difference in the number of sperm collected from females mated to brothers and unrelated males? I think that I may have missed the conditions that allow female control of paternity. In my experiment, females stayed one week with the first male and one week with the second male. This led to a situation where the first

male sired most of the offspring in all treatments, and larger females were more likely to carry offspring of two males. One possible explanation for this finding is that the first male filled the sperm storage sites of the female and only larger females had space for the second male's sperm. Thus if the mating period had been shorter, I might have seen an effect of relatedness on paternity success of males. In the seaweed fly (*Coelopa frigida*), females are able to bias paternity when they are mated to two males in quick succession but not when the time interval between matings is long (Blyth & Gilburn 2005). In any case, these results suggest that any female control of paternity must happen quite early on after insemination, possibly before sperm enter the storage sites, and it seems that females have no post-fertilisation measures, such as selective abortion, to bias paternity.

What about the effect of male relatedness on the number of sperm collected from females? Is that a male or a female effect? Unfortunately, a definite answer to this question cannot be given with my experimental set-up. The number of copulations males performed and the number of sperm they transferred was not controlled, and it is thus possible that unrelated males invested more in copulations with unrelated females and therefore we retrieved more sperm out of unrelated females.

Males are selected to invest different amounts of sperm in matings with different kinds of females, depending on their expected returns in each case (Wedell et al. 2002; Engqvist & Reinhold 2006). Differential sperm investment has been found in another poeciliid fish, *Poecilia mexicana*, where males invested less sperm in matings with asexual *P. formosa* females than in matings with conspecifics (Schlupp & Plath 2005). This differential investment was also visible in male reproductive behavior. Males also attempted copulations more often with conspecifics than with *P. formosa* females (Schlupp & Plath 2005). Here I found no evidence that males would have behaved differently towards their siblings and non-siblings in the pre copulatory male choice test. In fact, males spent more time in a sneaking position looking for a suitable moment for a sneak copulation with their siblings than non-siblings during the mating trials in a no-choice situation.

In the least killifish, females should be the choosier sex in terms of inbreeding avoidance, because females make a substantial investment in the developing embryos (Fraser & Renton 1940; Scrimshaw 1944; Grove & Wourms 1991) whereas males only provide sperm (Farr 1989). Hence it seems likely that the difference in sperm numbers that I found between sibling and non-sibling matings is a result of female decisions rather than male decisions. In addition to this theory-based argument, male behaviour also suggests that they do not avoid inbreeding.

This study shows that measuring only paternity success or pre-copulatory behaviour may not tell the whole story about inbreeding avoidance. Decrease in sperm

numbers after sibling matings, but no relatedness effect after fertilisation, suggests a conflict over inbreeding. Possibly, females try to avoid inbreeding by decreasing the amount of sibling sperm, but they are not able to bias paternity since males, at least in laboratory conditions, are very successful at performing sneak copulations.

5.3 Males prefer small females

So far in all Poeciliidae species studied, male preference for large females has been found (see introduction). Unexpectedly, I found that least killifish males prefer to interact with small females instead of large ones in a dichotomous male choice test, even though large females are more fecund (Schraeder & Travis 2008). I suggest that this unique preference for small females results from strong first male sperm precedence in this species (see section 5.2, inbreeding avoidance). Smaller females are younger and therefore more likely to be virgin, which probably makes them more profitable mates for males. However, during the free-swimming choice experiment, in which a male was freely interacting with both a small and a large female simultaneously, males did not discriminate between females based on their size. Males did not try to copulate more often with small females than large ones or follow small females longer than large ones. Even though the results of these two tests differ, they both suggest males do not prefer large females in this species, as they do in other poeciliids studied in this respect (see Introduction).

When presented with a virgin and a mated female of similar size, males showed no preference for either type. In some species of poeciliids, for example *P. latipinna* (Ptacek & Travis 1997) and the guppy (Herdman et al. 2004), males exhibit gonopore nipping behaviour possibly to assess female sexual pheromones and to detect female post-partum state. This behaviour is not shown by the least killifish. As the least killifish superfetates, it does not have a post-partum non-pregnant state like non-superfetating poeciliids do. Therefore, there may not have been selection on males to develop the ability to assess female mating status based on pheromone or other cues, and hence not the ability to distinguish virgin females from non-virgin females. Instead, the least killifish males may use female size as a proxy for virginity.

6. Unresolved issues and suggestions for future studies

It turned out to be surprisingly difficult to induce multiple paternity in the least killifish. The proportion of females producing multiply sired offspring was the highest (8 out of 12, 67 %) when females spent 30 days with three males in the "diverse *versus* related broods" experiment (**I**). When females were sequentially mated to two males, the proportion of multiply sired litters varied from 8 % (**I**) to 20 % (**III**). Even when females were sequentially mated to four males, only 20 % produced offspring of two males (**I**). I never detected

more than two sires among offspring of a single female. These results are in accordance with the amount of multiple paternities found in nature (Soucy & Travis 2003). The proportion of females carrying offspring of multiple males varies from 15 % to 66 % in different populations, and it is positively correlated with population density (Soucy & Travis 2003). Soucy and Travis (2003) did not report any cases of litters sired by more than two males. Thus it seems that the low degree of multiple paternity is characteristic for this species and not an artefact of my experimental designs.

The level of multiple paternity is lower in the least killifish than in many other poeciliid fishes (see Soucy & Travis (2003) for a comparison). Why this is the case has not been resolved. The least killifish differs from most poeciliids in that it is matrotrophic and it superfetates. Both of these traits make it more vulnerable to parent-offspring conflict over maternal provisioning as compared to most poeciliids, because offspring may have access to maternal resources and a large number of offspring simultaneously share the maternal environment. Because multiple paternity may lead to a conflict over resource allocation between offspring and therefore to low offspring quality, females may have benefited from the ability to control paternity or sperm storage to avoid or at least decrease this conflict. Unfortunately, the existence of this conflict could not be unequivocally demonstrated in my thesis. There is evidence in this direction, but further studies are needed to verify this. The interpretation of a conflict between offspring of multiple males is currently weakened by the fact that multiply sired litters at birth were so few. It might be worthwhile to check whether artificial insemination would induce higher rates of multiple paternity.

The way in which females might control paternity of their offspring is an open question. My finding that fewer sperm could be retrieved from females mated to brothers, compared to females mated to unrelated, males warrants further studies. Controlling the number of sperm inseminated through artificial insemination and then measuring paternity success of brothers and unrelated males would shed more light on the question whether it is the female or the male that is behind the effect on sperm numbers. If females inseminated with a mixture of sperm from a brother and an unrelated male produced more offspring of unrelated males, it would mean that females may have a way to control paternity. In that case, a likely mechanism of cryptic female choice would be sperm-female interaction (Bishop 1996; Bishop et al. 1996). On the other hand, if the mechanism of cryptic female choice were the ejection of unwanted sperm (Pizzari & Birkhead 2000), there probably would be no difference in paternity success of related and unrelated males when sperm mixtures are used.

Another possible mechanism of female control may be delayed offspring production. Females mated to one random male very rarely produced offspring. That

was not only obvious in the multiple mating experiment (**I**) (table 3), but during the whole thesis work when my aim was to produce families for different experiments. However, when the time a pair of fish were together increased, pregnancy success also increased (see **II**). It was therefore very surprising that there is such a strong first male sperm precedence in this species (**III**).

7. Conclusions

The main question of this thesis was "why do females mate with more than one male?" My answer based on my results is "because they cannot avoid it". Of course, females mated to more than one male were more likely to become pregnant, which is clearly a major benefit. How that relates to the mating system of the least killifish is less clear. Presumably females do not have much control over mating in this species as males mainly rely on sneak copulations (Bisazza & Pilastro 1997). However, the success rate of sneak copulations is very low (4 to 20 %, Bisazza & Pilastro 1997; Aspbury & Basolo 2002). It then seems likely that a female gets successful copulations from a random subset of males, unless she has a way to avoid unwanted copulations. Thus a female is likely to get copulations from more than one male even without actively searching for them.

I believe that the least killifish females actively avoid producing offspring of multiple males, because it leads to a decrease in offspring quality in terms of longer times to reach sexual maturity (**I**). One explanation for this might be an increased conflict between offspring of multiple males over maternal provisioning.

I did not find clear support for the hypothesis that females would be polyandrous in order to avoid inbreeding (**III**), but I found an inbreeding avoidance mechanism in this species. Less sperm was retrieved from females mated to brothers as compared to females mated to unrelated males. It was not surprising to find an inbreeding avoidance mechanism in the least killifish as this species suffers from strong inbreeding depression in male mating behaviour and in two important life history traits (**II**). Population sizes of the least killifish often collapse during droughts and recover slowly (Ruetz et al. 2005). During re-colonisation, mating between relatives is quite likely to occur (Ruetz et al. 2005), which may have selected for the evolution of inbreeding avoidance mechanisms.

I also found that males are choosy (**IV**). Males are expected to invest more in matings with high quality females, even if their investment in each mating is small (Parker 1983). The trait signalling high female quality may be for example her size, age or probability of sperm competition. How males assess these qualities depends on the mating system and natural history of the species in question. In contrast to earlier studies on Poeciliidae, male preference for large females was not found. Instead, males preferred to interact with small females in a dichotomous choice test. Because smaller females are more likely to be virgin (as they are younger) and the first male to mate sires

most of the offspring, I suggest that males view smaller females as more profitable mating partners.

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